Diverse mitotic and interphase functions of condensins in Drosophila

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Diverse Mitotic and Interphase Functions of Condensins in Drosophila

Neville Cobbe, Ellada Savvidou and Margarete M. S. Heck[1]

Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh EH9 3JR, United Kingdom

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ABSTRACT

The condensin complex has been implicated in the higher-order organization of mitotic chromosomes in a host of model eukaryotes from yeasts to flies and vertebrates. Although chromosomes paradoxically appear to condense in condensin mutants, chromatids are not properly resolved, resulting in chromosome segregation defects during anaphase. We have examined the role of different condensin complex components in interphase chromatin function by examining the effects of various condensin mutations on position-effect variegation in *Drosophila melanogaster*. Surprisingly, most mutations affecting condensin proteins were often found to result in strong enhancement of variegation in contrast to what might be expected for proteins believed to compact the genome. This suggests either that the role of condensin proteins in interphase differs from their expected role in mitosis or that the way we envision condensin's activity needs to be modified to accommodate alternative possibilities.

CONDENSINS are multi-subunit protein complexes that are conserved in all eukaryotes for which adequate sequence data are available, where they play vital roles in mitotic chromosome assembly and segregation as well as in influencing interphase processes such as gene silencing and checkpoint responses (Hirano 2005). Each condensin complex contains a heterodimer of SMC2 and SMC4 proteins, both of which are members of the highly conserved *s*tructural *m*aintenance of *c*hromosomes (SMC) family (Cobbe and Heck 2004), typically in addition to three regulatory non-SMC proteins. However, there may be at least two different condensin complexes among various eukaryotes, each containing a unique set of non-SMC subunits (Hagstrom and Meyer 2003; Ono *et al.* 2003). In vertebrates, the condensin II complex appears to participate in an early stage of chromosome condensation within the prophase nucleus, whereas the condensin I holocomplex appears to assemble on mitotic chromosomes only after nuclear envelope breakdown and is thought to cooperate with condensin II to facilitate metaphase chromosome compaction and sister-chromatid resolution (Hirota *et al.* 2004; Ono *et al.* 2004).

In addition to influencing mitotic chromosome structure, condensin proteins are known to play roles during interphase (Hagstrom and Meyer 2003). A functional link between mitotic chromosome condensation and global regulation of gene expression was initially demonstrated in *Caenorhabditis elegans* on the basis of the involvement of an SMC2/4 heterodimer in dosage compensation. Transcription from each of the X chromosomes is reduced twofold in hermaphrodites (XX) of this organism to match the level of X-linked gene expression in males (XO). The discovery that a variant SMC4-type protein (DPY-27) is an essential regulator of dosage compensation (through its association with the X chromosome) provided the first clue that SMC proteins might be involved in this process (Chuang *et al.* 1994, 1996). Subsequently, MIX-1 was identified as an SMC2-type protein required for both mitosis and dosage compensation, whose restricted localization to the X chromosome was shown to depend on that of DPY-27 (Lieb *et al.* 1998). The dosage compensation complex also consists of at least two non-SMC subunits, including DPY-26 and DPY-28 (Lieb *et al.* 1996; Hagstrom and Meyer 2003). On the other hand, the mitotic function of MIX-1 requires its association with a more conventional SMC4-type protein (Hagstrom *et al.* 2002). It has therefore been suggested that MIX-1 may have been enlisted to the dosage compensation complex through the evolution of DPY-27 into a highly specialized SMC protein, altering the higher-order structure of X chromosomes by a mechanism potentially related to that involved in mitotic chromosome condensation.

Strikingly, the same dosage compensation complex is also recruited to the autosomal *her-1* gene (a male sex-determination gene) to repress its transcription >20-fold and thereby elicit hermaphrodite differentiation (Chu *et al.* 2002). This therefore demonstrates a link between the roles of condensin SMC proteins in gene-specific and chromosome-wide transcriptional

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[1]*Corresponding author:* Wellcome Trust Centre for Cell Biology, University of Edinburgh, School of Biological Sciences, Institute of Cell Biology, Michael Swann Bldg., Mayfield Rd., Edinburgh EH9 3JR, United Kingdom. E-mail: margarete.heck@ed.ac.uk

repression. Various non-SMC condensin subunits also appear to be required for transcriptional silencing in both *Saccharomyces cerevisiae* (Bhalla *et al.* 2002) and *Drosophila melanogaster* (Lupo *et al.* 2001). However, it remains to be seen if these proteins function during interphase as part of the canonical condensin complex or other alternative SMC-containing complexes. Similarly, Cnd2/CAP-H (a non-SMC condensin subunit) was recently shown to be required for the activity of Cds1 (a G_2 checkpoint kinase) in *Schizosaccharomyces pombe* (Aono *et al.* 2002). Although all the other condensin components in *S. pombe* were also required for full Cds1 activity, the condensin SMC proteins (Cut3 and Cut14) differed from Cnd2/CAP-H as they were not required for viability in the presence of hydroxyurea during S phase (Aono *et al.* 2002). The contrasting interphase requirements for different condensin proteins in *S. pombe* therefore suggest that these proteins also may not necessarily act in the same complex during interphase as during mitosis.

In this article, we further explore the varied activities of condensin proteins in *D. melanogaster* by analyzing multiple alleles of *gluon*, encoding the Drosophila ortholog of SMC4, together with various mutations affecting other condensin components. A large number of *gluon* alleles were previously generated through imprecise excision of the *P* element in glu^{1} (Figure 1A), resulting in additional embryonic lethal lines (glu^{17C}, $glu^{88\text{-}37}$, and $glu^{88\text{-}41B}$) displaying chromosome segregation defects similar to those described in $glu^{88\text{-}82}$ mutant embryos in which chromatin bridges were most frequently observed (Steffensen *et al.* 2001). We have therefore exploited these different *gluon* alleles, together with mutations affecting other components of the condensin I complex in Drosophila (Savvidou *et al.* 2005), to explore possible functions of condensins in interphase as well as in mitosis. These include the embryonic lethal $barr^{L305}$ allele, which is considered to be a null mutation of the *barren* gene (Bhat *et al.* 1996) encoding the Drosophila ortholog of the CAP-H subunit (Hirano *et al.* 1997) and the embryonic lethal $red\ sea^{64}$ mutant (Philp 1998), which was subsequently shown by complementation analysis to be allelic to the Drosophila gene encoding CAP-G (referred to hereafter as the $dcap\text{-}g^{64}$ allele). Finally, a larval lethal mutation affecting SMC2, known as $smc2^{jsl2}$, was discovered when a collection of EMS mutants generated by Jeff Sekelsky (University of North Carolina at Chapel Hill) was screened using an antibody raised against SMC2 in Drosophila (Savvidou *et al.* 2005). By examining the effect of each of these mutations on position-effect variegation (PEV) at different loci, we demonstrate further effects of different condensin components in modulating interphase gene expression (with the exception of CAP-D2, for which there are currently no mutants available) in addition to regulating the structure and behavior of mitotic chromosomes.

MATERIALS AND METHODS

Creation of a *gluon;prospero* double-mutant line: A multiply balanced $L^2/CyO;\ MRS/TM6B$ stock was initially crossed with $+/+;\ pros^{17}/TM6B$ flies. The L^2 and *CyO* progeny were collected and crossed with each other to generate $L^2/CyO;\ pros^{17}/MRS$ flies. These were then crossed against the multiply balanced $glu^1/CyO;\ MRS/TM6B$ line and the $glu^1/CyO;pros^{17}/TM6B$ progeny were collected. These were finally crossed with $In(2LR)Gla,\ wg^{Gla\text{-}1},\ Bc^1/T(2;3)TSTL$ flies to create a stock of $glu^1;pros^{17}/T(2;3)TSTL$ flies.

Selection of homozygous mutant animals: Embryonic lethal condensin mutations were maintained over *CyO* balancer chromosomes expressing GFP under control of the *Krüppel* gene (Casso *et al.* 2000) and homozygous mutant embryos were selected on the basis of the absence of fluorescence in the amnioserosa or the Bolwig's organs by sorting in halocarbon oil, as described (Steffensen *et al.* 2001; Savvidou *et al.* 2005). As the *gluon* and *prospero* genes are on the second and third chromosomes, respectively, the double mutants were maintained over *T(2;3)TSTL*, a translocation balancer chromosome allowing simultaneous isolation of homozygotes at both loci. The homozygous $glu^1;pros^{17}$ mutant embryos were then identified on the basis of the elevated number of mitotic nuclei in their central nervous system (resulting from the $pros^{17}$ mutation). As a control, the $pros^{17}$ mutation was balanced over the *TM3*-5 balancer chromosome expressing GFP under control of the *Krüppel* gene (Casso *et al.* 2000) and $pros^{17}$ homozygous embryos were selected on the basis of their lack of GFP expression. The larval lethal $smc2^{jsl2}$ allele was maintained over a *CyO-GFP* balancer and homozygous larvae were similarly selected on the basis of the absence of GFP fluorescence.

Fixation of embryos and larval brains: A fast formaldehyde fix was used to preserve cortical cytoskeletal structures such as microtubules on the basis of protocols described elsewhere (Therkauf and Heck 1999; Rothwell and Sullivan 2000). Dechorionated embryos were promptly rinsed in filter-sterilized EBR (Ephrussi Beadle Ringer's solution; 130 mm NaCl, 4.7 mm KCl, 1.9 mm $CaCl_2$, 10 mm Hepes, pH 6.9) and flushed into a small glass vial using heptane. The embryos were then fixed in equal volumes of heptane and 40% EM-grade formaldehyde (Electron Microscopy Sciences, Fort Washington, PA) with extremely gentle rocking for 3 min, ensuring that the embryos were spread in a monolayer at the interface between the two phases. The lower formaldehyde phase was then removed carefully using a long-tipped glass Pasteur pipette and replaced with an equal volume of Merck ARISTAR methanol to remove the vitelline membrane. The vial was capped again, shaken vigorously, vortexed for 15 sec, and then left to stand upright for 1 min. The devitellinized embryos that had sedimented to the bottom of the vial were removed using a long-tipped glass Pasteur pipette and transferred to a 1.5-ml microcentrifuge tube. The methanol was replaced with fresh Merck ARISTAR methanol four times to remove any remaining formaldehyde or heptane and mixed after each replacement. Fixed embryos were stored in methanol at −20° before processing for immunofluorescent detection as described (Therkauf and Heck 1999; Rothwell and Sullivan 2000). Developmental staging of embryos was assessed according to the numbering scheme introduced by Mary Bownes (Bownes 1975, 1982) and later refined by Eric Wieschaus and Christiane Nüsslein-Volhard (Wieschaus and Nüsslein-Volhard 1998). Brains from third instar larvae were fixed and processed for immunofluorescence as described (Bonaccorsi *et al.* 2000; McHugh *et al.* 2004).

Antibodies: Primary antibodies were used for immunofluorescence or immunoblotting as follows: α-tubulin (mouse monoclonal antibody B5-1-2 used at 1:1000; Sigma, St. Louis),

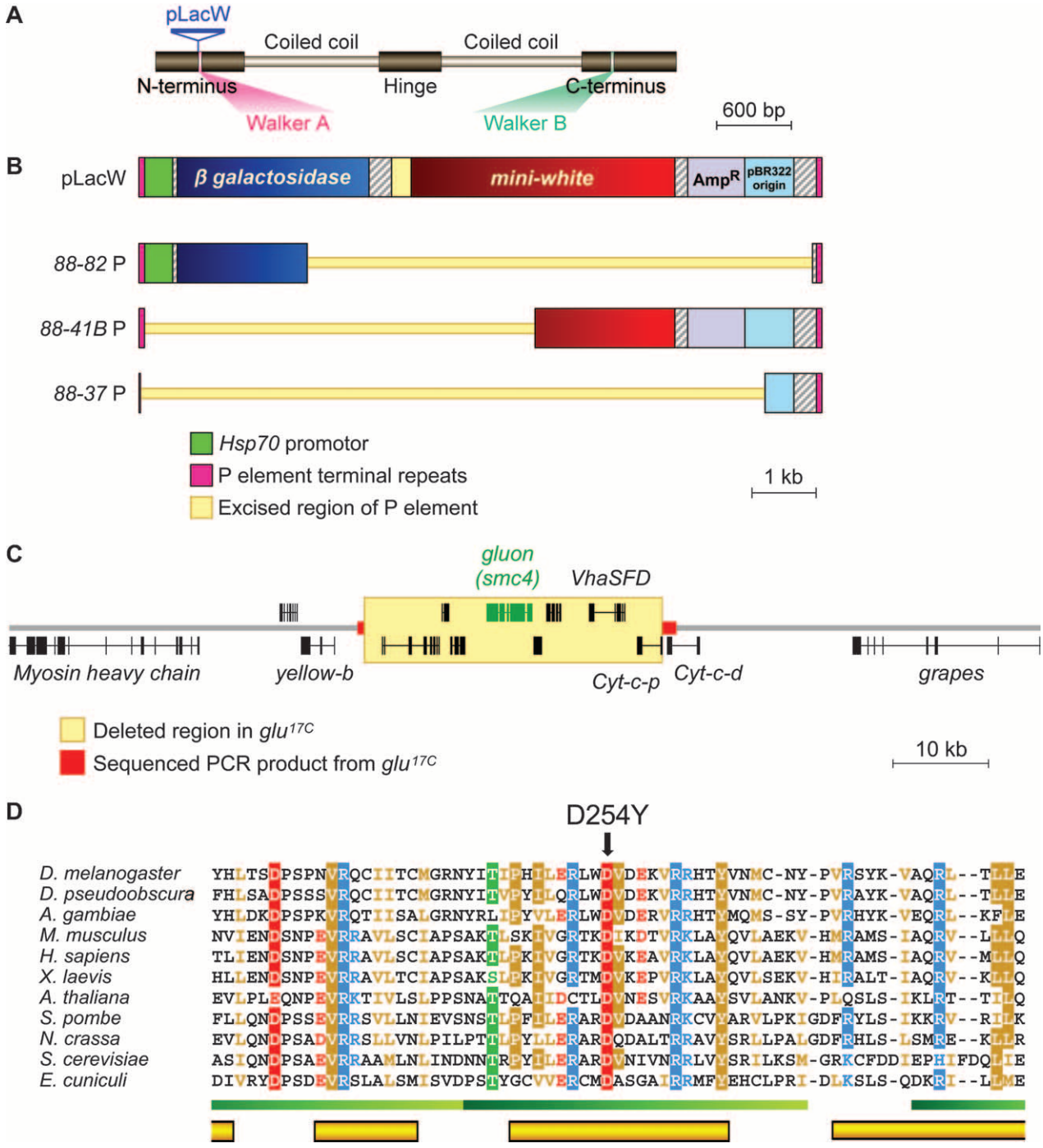

FIGURE 1.—Molecular characterization of embryonic lethal condensin mutants. (A) Domain structure of SMC4 mapped onto the *gluon* coding sequence, showing the position of the glu^{1} *P* element (not to scale) just upstream of nucleotides 352–375 corresponding to the Walker A motif residues 118–125. (B) Map of the *pLacW P* element inserted in glu^{1} and the remaining *P*-element sequence in the $glu^{88\text{-}82}$, $glu^{88\text{-}41B}$, and $glu^{88\text{-}37}$ *P*-element excision alleles. (C) Map showing position of the *gluon* gene and surrounding genes on chromosome 2L. The large boxed area surrounding SMC4 shows the genes deleted in glu^{17C}. The red boxed area denotes the region amplified by PCR and sequenced to confirm the lesion. (D) Alignment of residues 220–285 of dCAP-G with CAP-G orthologs from different species, highlighting the position of the D254Y substitution in $dcap\text{-}g^{64}$ in a conserved region of the protein. Conserved residues are displayed in color (acidic residues in red, basic residues in blue, hydrophilic residues in green, and hydrophobic residues or conserved glycines in ochre), with the most conserved or invariant residues indicated by boxed shading. The predicted locations of HEAT repeats are shown by shaded green lines below the alignment, while predicted α-helices are shown by shaded yellow boxes.

phosphohistone H3 (P-H3; rabbit polyclonal used at 1:500; Upstate Biotechnology, Lake Placid, NY), P-H3 (mouse monoclonal used at 1:500; New England Biolabs, Beverly, MA), lamin (rabbit polyclonal used at 1:500; provided by Paul Fisher, State University of New York, Stony Brook, NY), Barren rabbit polyclonal used at 1:500 (Bhat *et al.* 1996), SMC2 rabbit polyclonal used at 1:500 (Savvidou *et al.* 2005), SMC4 rabbit polyclonal used at 1:500 (Steffensen *et al.* 2001), SCC1 rabbit polyclonal used at 1:500 (Warren *et al.* 2000), and CAP-D2 rabbit polyclonal used at 1:10,000 for immunoblotting (Savvidou *et al.* 2005). All fluorescent or horseradish-peroxidase (HRP)-conjugated secondary antibodies (Molecular Probes, Eugene, OR; Jackson ImmunoResearch Laboratories, West Grove, PA; and Amersham Biosciences, Buckinghamshire, UK) were used according to the manufacturer's instructions.

TUNEL labeling of staged embryos: Combined antibody staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) of staged embryos was performed using the Appligene Oncor Apotag kit, with modifications detailed below. Fixed embryos were permeabilized for 1 hr in PBS with 1% Triton X-100 and washed for 2 min in PBSTx (PBS with 0.05% Triton X-100) until all samples were ready for the Apotag reaction. The PBSTx was removed by careful aspiration and at least 100 μl equilibration buffer per sample was applied for 2 min. The buffer was then removed and at least 100 μl of working terminal deoxynucleotidyl transferase (TdT) enzyme solution (150 μl TdT enzyme with 350 μl reaction buffer) was immediately applied before the embryos were incubated for 1 hr at 37° with rotation on a mixer. The embryos were then washed in stop/wash solution with gentle agitation for 15 sec and incubated for 10–15 min. The embryos were washed three times for 10 min in PBSTx and blocked in PBS + 3% bovine serum albumin (Sigma) for 30 min at room temperature. They were next washed for 5 min in PBSTx and incubated overnight at 4° in mouse antidigoxigenin antibody (Boehringer, Indianapolis) diluted 1:100 in PBSTx. The embryos were then washed six times for 5 min each time before performing any additional antibody incubations as described earlier or staining of DNA with 0.05 μg/ml DAPI. The mouse antidigoxigenin antibody was detected using an Alexa Fluor 594 conjugated anti-mouse antibody (Molecular Probes).

Microscopy: All preparations were examined with an Olympus Provis microscope, equipped with epifluorescence optics. Images were captured with an Orca II CCD camera (Hamamatsu, Bridgewater, NJ) and Smart Capture 2 software (Digital Scientific). Fixed embryos were mounted in 90% glycerol/10% PBS. Image analysis was performed using IPLab Spectrum version 3.1 and Adobe Photoshop version 4.0.

Preparation of larval brain samples for SDS-PAGE: Ten to 20 brains were suspended in 50 μl lysis EBR (EBR with 10 mm EDTA, 10 mm dithiothreitol, 1:100 dilutions of each of the protease inhibitors PMSF, CLAP, and Trasylol). The material was homogenized using a Kontes pestle and mini-motor immediately before adding 25 μl of warm (65°) 3× SDS-PAGE sample buffer with DTT (6% SDS, 150 mm trizma base, 30% glycerol, 0.03% bromophenol blue, 6 mm EDTA, 100 mm dithiothreitol). A hole was then pierced in the top of tube and it was heated at 100° for 10 min. Finally, the tube was spun briefly and the sample was loaded onto a polyacrylamide gel or stored at −20°.

Immunoblotting: Protein extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH) in a trans-blot apparatus. Membranes were blocked in PBS + 0.1% Tween 20 (PBSTw) and 5% semiskimmed milk for 1 hr at room temperature (RT) and then incubated for 1–1.5 hr with the primary antibody in PBSTw. After washing three times for 5, 15, and 10 min with PBSTw, the membranes were incubated in an HRP-linked secondary antibody for 1 hr in PBSTw at RT. Finally, the membranes were washed as above in PBSTw and immunocomplexes were detected by enhanced chemiluminescence (Amersham Biosciences). The nitrocellulose was then exposed to Kodak XAR-5 film for autoradiography and films were developed using a Konica SRX-101A developer.

PhosphorImager analysis: Samples were processed as described for immunoblotting but instead of using an HRP-conjugated secondary antibody, a Cy5-conjugated antibody (Jackson ImmunoResearch Laboratories) was used at a dilution of 1:200. The intensity of the signal was measured using a STORM 860 scanning phosphorImager and calculated using the ImageQuant program (Amersham). The intensity was finally normalized using α-tubulin as a loading control. Values were corrected by subtracting the background of the corresponding lane from each band.

Analysis of position-effect variegation: Male flies from each mutant line (balanced over *CyO*) were crossed against w^{m4h} virgin females at 25° and the resultant straight-winged male progeny were selected and allowed to age for 4 days after eclosion. The flies were then frozen at −80° and the heads were decapitated by vortexing. The fly heads were isolated by repeated sieving through nylon meshes and 100 fly heads were counted and placed in a 1.5-ml microfuge tube. The drosopterin pigments were extracted and analyzed by spectrophotometry as described (Ashburner 1989), using the heads of similarly treated w^{1118} flies as a blank. Crosses between *gluon* virgin females and male flies carrying *SUPor-P* transposon insertions on the Y chromosome (Yan *et al.* 2002) or between condensin mutant males and virgin females carrying *hsp70-*w^{+} transgenes on the fourth chromosome (Cryderman *et al.* 1999) were similarly performed as described above. Crosses with w^{1118} flies were used as controls for all reporters of PEV to generate hemizygotes in an otherwise wild-type background, thereby avoiding potential complications associated with background modifiers of PEV on balancer chromosomes (Wallrath and Elgin 1995).

Frequency distributions of variegated eye color in different mutant backgrounds were generated by photographing both eyes of individual 4-day-old male progeny against a uniform white background with an Olympus C-2020 Z digital camera. The level of pigmentation was then quantified using the histogram feature of Adobe Photoshop 4.0 to measure the mean pixel intensity of eye color in the images. To correct for any possible variations in ambient illumination during image capture, the luminosity of the traced eyes was subtracted from that of the white background. The resulting values were then rounded and a frequency distribution was plotted, using the ratio of the mean absorbance to the mean pixel intensity in each genetic background to calibrate the respective frequencies in terms of absorbance.

To examine the dominance relationships between mutant alleles identified as strong enhancers or suppressors of variegation, the *In(1)*w^{m4h} chromosome was crossed into each of the mutant lines using w^{m4h}*;L/CyO virgin* females. Reciprocal crosses between strong enhancers of variegation and strong suppressors of variegation were then performed and the absorbance of 100 heads from 4-day-old straight-winged male progeny was measured as described above.

RESULTS

Mitotic defects in *gluon* and *dcap-g* mutant embryos: As cells undergoing mitosis in $glu^{88\text{-}82}$ mutant embryos were shown to either accumulate in metaphase or progress to anaphase with chromatin bridges (Steffensen *et al.* 2001), we attempted to determine the ultimate fate

of these defective divisions in different *gluon* mutant alleles. One possible consequence might be that a metaphase delay would lead to an elevated mitotic index as fewer cells would be exiting mitosis, or a large number of defective mitotic divisions might lead to increased levels of apoptosis. These possibilities were tested by measuring the levels of apoptosis and mitosis after dual labeling of fixed embryos with TUNEL and immunofluorescent detection of histone H3 phosphorylated at serine 10 (Wei and Allis 1998). By counting the number of TUNEL-positive and phosphohistone-H3-positive cells in sections of staged embryos, we observed that *gluon* alleles have reduced levels of both mitosis and apoptosis (Figure 2A). Since cell losses are no longer regulated by compensatory divisions during these post-blastoderm mitotic cycles and supernumerary cells can be disposed of by apoptosis (Busturia and Lawrence 1994; Namba *et al.* 1997; Li *et al.* 1999), the ratio of mitosis to apoptosis should reflect the relative frequency of successful nuclear divisions. Indeed, we observed that the *gluon* mutant embryos have a lower ratio of mitosis to apoptosis compared to wild-type Canton-S embryos (Figure 2B). Furthermore, this mitosis/apoptosis ratio appeared to decrease as the mutants aged (in contrast to wild-type embryos), with the most extreme trends observed in homozygous glu^{17C} embryos. We therefore concluded that mutations affecting SMC4 result in both decreased mitotic activity and an increased number of defective mitotic divisions. However, the reduced mitotic index itself does not seem to be a consequence of elevated absolute levels of apoptosis, suggesting either that the mutant cells unable to properly resolve their chromosomes might somehow reverse out of mitosis (Rieder and Cole 1998) or that the number of cells initially able to enter mitosis is reduced. As there is relatively little mitotic activity in either stage 14 or stage 15 mutant embryos, the mitotic phenotypes of *gluon* mutant alleles were therefore examined in a genetic background with elevated mitotic activity.

Unlike the previously described $glu^{88\text{-}82}$ allele, mitotic phenotypes were less frequently observed in the original glu^{1} *P*-element insertion allele and most other excision alleles, even though the frequency of mitotic nuclei was similar in $glu^{88\text{-}82}$ and glu^{1} homozygous embryos. By contrast, *barren* (Bhat *et al.* 1996) and *dcap-g* (Dej *et al.* 2004; Jäger *et al.* 2005) mutants display chromosomal defects in mitotic domains during cycles 15 or 16 (Bownes' stage 11). However, SMC4 is highly expressed in Drosophila ovaries (Steffensen *et al.* 2001) and maternally loaded stockpiles of the protein persist throughout embryogenesis, such that phenotypes are not observed in any embryonic lethal *gluon* mutants until stage 14. If the relative lack of mitotic phenotypes observed in most *gluon* mutants is due to the slower turnover of SMC4 compared to Barren and dCAP-G, then increasing the number of mitotic divisions should locally deplete maternal SMC4 more rapidly and allow

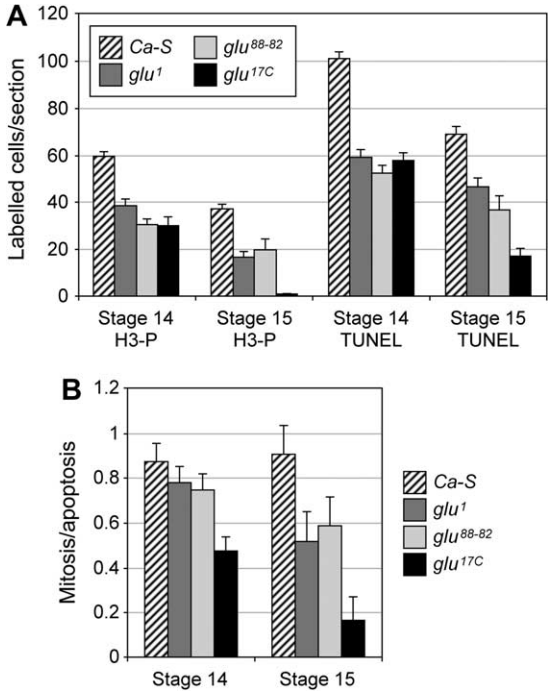

Figure 2.—Altered levels of mitosis and apoptosis in *gluon* mutant embryos. (A) Counts of cells positive for TUNEL or staining with an antibody to histone H3 phosphorylated at serine 10, showing the number of labeled cells per lateral section for wild-type *Canton-S* (*Ca-S*; 353 embryos), glu^{1} (167 embryos), $glu^{88\text{-}82}$ (186 embryos), and glu^{17C} (175 embryos). (B) The ratio of mitotic cells to apoptotic cells in wild-type and *gluon* homozygous mutant embryos at different stages of embryogenesis.

mutant phenotypes to be observed more readily. This was achieved by introducing a mutation in *prospero*, which encodes a pan-neural transcription factor that normally inhibits cell proliferation during neuroblast divisions by repressing transcription of several cell-cycle regulatory genes, such as *cyclin A*, *cyclin E*, and *string*, the Drosophila homolog of *cdc25* (Li and Vaessin 2000). The original glu^{1} mutation was therefore combined with $pros^{17}$, an amorphic allele resulting from a small deletion within the *prospero* locus (Gertler *et al.* 1993), and mitotic chromosomes from $glu^{1};pros^{17}$ homozygous embryos were compared with $pros^{17}$ controls.

As with $glu^{88\text{-}82}$ homozygotes, no easily discernible effect was observed before metaphase in $glu^{1};pros^{17}$ mutant embryos, although 11% of metaphase figures appeared abnormal, containing more than the usual chromosome complement (Figure 3A). These aneuploid or polyploid metaphases presumably resulted from segregation defects in a previous mitotic cycle and are consistent with the giant aneuploid nuclei observed when SMC4 is depleted by RNA interference (RNAi) in *C. elegans* (Hagstrom *et al.* 2002). Furthermore, chromatin bridges were observed in $glu^{1};pros^{17}$ mutant embryos at anaphase and telophase (Figure 3C) with similar frequencies to those previously observed in $glu^{88\text{-}82}$ homozygotes (Steffensen *et al.* 2001). Therefore, the high frequency of segregation defects observed

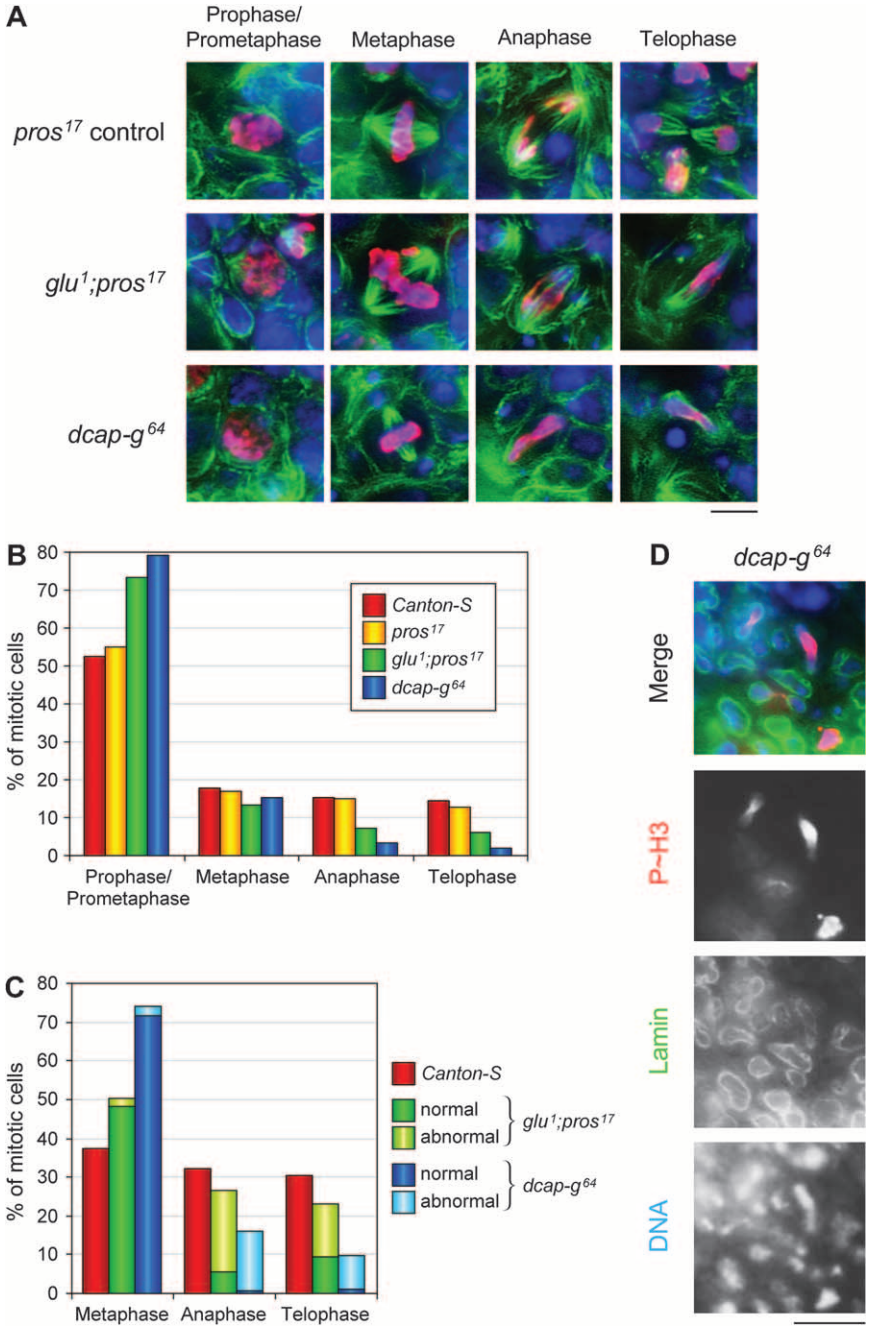

FIGURE 3.—Mitotic phenotypes of embryonic lethal condensin mutants. (A) Mitotic figures of homozygous *pros*^{17} embryos (top), *glu*^{1};*pros*^{17} double-mutant embryos (middle), and *dcap-g*^{64} embryos (bottom). Immunofluorescent labeling of embryos was performed with antibodies detecting mitotic phosphorylation of histone H3 on serine 10 (P-H3, red) and α-tubulin (green), while DNA was stained with DAPI (blue). Bar, 5 μm. (B) Quantitation of mitotic parameters in wild-type (*Canton-S*, 834 nuclei) and mutant embryos. No defects in mitotic progression are apparent in homozygous *pros*^{17} embryos (1511 nuclei), whereas the condensin mutants appear to accumulate in prophase/prometaphase (1846 *glu*^{1};*pros*^{17} nuclei and 2592 *dcap-g*^{64} nuclei). (C) Quantitation of mitotic defects in *Canton-S* (397 nuclei) and condensin mutant embryos (492 *glu*^{1};*pros*^{17} nuclei and 537 *dcap-g*^{64} nuclei). In addition to a high frequency of anaphase and telophase chromosome bridges, the condensin mutants also appear to delay in metaphase. (D) Immunofluorescent labeling of homozygous *dcap-g*^{64} embryos with antibodies detecting P-H3 (red) and lamin (green), with DAPI staining of DNA shown in blue in the merged image. Bar, <10 μm.

in *glu*$^{88\text{-}82}$ mutant embryos can be reproduced by increasing the mitotic activity of *glu*^{1} mutants and presumably depleting wild-type SMC4 levels more quickly than in *glu*^{1} mutants alone. Previously, we described how the proportion of mitotic cells in metaphase was significantly greater in embryos homozygous for the *glu*$^{88\text{-}82}$ allele (STEFFENSEN *et al.* 2001). Although somewhat obscured by the greater proportion of cells in prophase/prometaphase when all mitotic stages are represented (Figure 3B), we were similarly able to detect a twofold increase in the proportion of mitotic cells in metaphase relative to anaphase in *glu*^{1};*pros*^{17} mutant embryos and a more than fourfold increase in the proportion of metaphase figures in *dcap-g*^{64} embryos (Figure 3C), in contrast to wild-type or *pros*^{17} controls in which the proportion of metaphase and anaphase figures is roughly equal.

In contrast to most *gluon* mutant alleles, mitotic defects were more readily observed in *dcap-g*^{64} embryos as similarly aged mutant homozygotes appeared to delay at earlier developmental stages with higher mitotic activity (Bownes' stages 11–13). As shown in Figure 3A, *dcap-g*^{64} homozygous embryos also displayed obvious chromatin bridges in anaphase and telophase, in addition to demonstrating an even greater accumulation of mitotic cells at prophase/prometaphase and metaphase than observed in *gluon* mutants (Figure 3, B and C). As previously described for various condensin mutants

(Steffensen *et al.* 2001; Dej *et al.* 2004), we consistently observed P-H3 persisting most strongly on the lagging chromatid arms or chromatin bridges in telophase. When *dcap-g*^{64} homozygous mutant embryos were labeled with antibodies against lamin, we also observed delayed lamin reassembly around chromatin bridges (Figure 3D), suggesting that the failure in anaphase chromosome segregation may delay dephosphorylation events concomitant with mitotic exit.

Primers flanking the original *P*-element insertion site in the *glu*^{1} allele were used to amplify the remaining *P* element from homozygous mutant embryos. These PCR products were then sequenced and the nature of the lesion in each of these mutant alleles was determined (Figure 1). Like the original *glu*^{1} allele, each of these excision alleles contained stop codons in all reading frames at the 5′-end of the *P* element, so the mutants are predicted to be nulls. In each of these mutant alleles, a 122-residue truncated protein containing only the first 119 amino acids of SMC4 could be produced, lacking even the Walker A motif (Figure 1A). The sequence for the molecular lesions explains why the red eye color resulting from the *mini-white* transgene is absent in each of these excision alleles, as the entire coding sequence is deleted in most alleles (with the exception of *glu*$^{88\text{-}41B}$, in which only the 3′ half of the *mini-white* transgene is present). Furthermore, the sequence data also suggest that the *glu*$^{88\text{-}82}$ allele might be a neomorph in which the *Hsp70* promoter could possibly drive expression of a truncated SMC4 protein containing amino acids 195–1409. However, we have been unable to verify the presence of such a truncated protein as previously generated antibodies were raised against an N-terminal fragment, corresponding to amino acids 80–303 of wild-type SMC4 (Steffensen *et al.* 2001). Sequencing of the molecular lesion in *glu*^{17C} revealed a large deletion of ~31 kb, resulting in a knockout for SMC4. Although a number of other genes were also removed by the deletion, the *glu*^{17C} allele was found to affect PEV in the same way as most other *gluon* alleles (as described below).

Sequencing of the *dcap-g*^{64} allele confirmed that the gene encoding CAP-G was indeed affected in these mutants, revealing a G954T transversion that results in a D254Y substitution. This mutation is significant as it affects a residue that is invariant among CAP-G sequences from different organisms and is found in a predicted HEAT repeat in the most conserved region of the protein. The discovery of HEAT repeats in CAP-G and CAP-D2 orthologs from *S. pombe*, *S. cerevisiae*, and Xenopus was previously thought to signify possible regions of interaction between condensin proteins (Neuwald and Hirano 2000), as these tandemly repeated bihelical structures are thought to play roles in protein-protein interactions (Andrade and Bork 1995). Indeed, such interactions were subsequently confirmed by co-immunoprecipitation experiments in which the HEAT-repeat-containing N-terminal moiety of CAP-D2 was shown to be required for interactions with other condensin proteins while the CAP-D2 C-terminal region interacted specifically with CAP-G (Ball *et al.* 2002). We therefore speculate that the replacement of an invariant aspartate residue with a tyrosine in a highly conserved region of CAP-G may disrupt a salt bridge required for formation of the bihelical HEAT repeat structure, thereby rendering the protein unable to interact normally with other condensin subunits.

Mitotic defects in *smc2* mutant larvae: We discovered a larval lethal mutation affecting SMC2, known as *smc2*^{jsl2}, among a collection of EMS mutants after immunoblotting larval protein extracts with an antibody generated against the N-terminal region (amino acids 24–309) of SMC2 (Savvidou *et al.* 2005) to detect lines in which the protein showed reduced expression or altered mobility. Unlike the previously characterized condensin mutant alleles, we did not detect mitotic phenotypes in *smc2*^{jsl2} embryos and the observed hatching frequencies were not consistent with embryonic lethality. However, the brains in *smc2*^{jsl2} homozygous third instar larvae were much smaller than either wild-type or heterozygous brains (Figure 4B), suggesting a proliferation defect. When third instar larval brains from heterozygous (control) and homozygous larvae were stained for phosphorylated histone H3, α-tubulin, and DNA, the percentage of mitotic cells in *smc2*^{jsl2} homozygous mutant brains (0.3%) was found to be significantly lower than in heterozygous or wild-type controls (1.6%). Similarly, the percentage of mitotic cells in younger second instar *smc2* ^{jsl2} homozygous mutant brains was only 0.4%, compared to 1.3% in controls. Analysis of the few mitotic cells that were present in homozygous brains showed several defects in chromosome morphology. Chromosomes often appeared fuzzy with almost no sister-chromatid resolution while cells in telophase showed extensive chromatin bridges (Figure 4A). However, the stage of many mitotic cells (up to 56% in the case of third instar mutant larval brains) could not be easily determined as the chromosome structure was so abnormal, whereas cells from control heterozygous brains showed normal chromosome morphology and segregation (Figure 4A). All telophase- or anaphase-like cells in *smc2*^{jsl2} homozygotes had chromosome bridges. Furthermore, 2.7% of interphase cells in *smc2*^{jsl2} homozygotes were also joined by chromosome bridges (forming apparently binucleate or trinucleate cells), compared to 0.1% in control brains, presumably reflecting defective chromosome segregation and failed cytokinesis in previous mitotic cycles. Nevertheless, no obvious delays in mitotic progression were apparent in *smc2*^{jsl2} mutant brains, although quantitation was limited by the significantly reduced mitotic index and not easily classifiable mitotic figures.

The mutation in *smc2*^{jsl2} was identified by sequencing the *smc2* locus from homozygous second instar larval genomic DNA, revealing a premature stop codon at

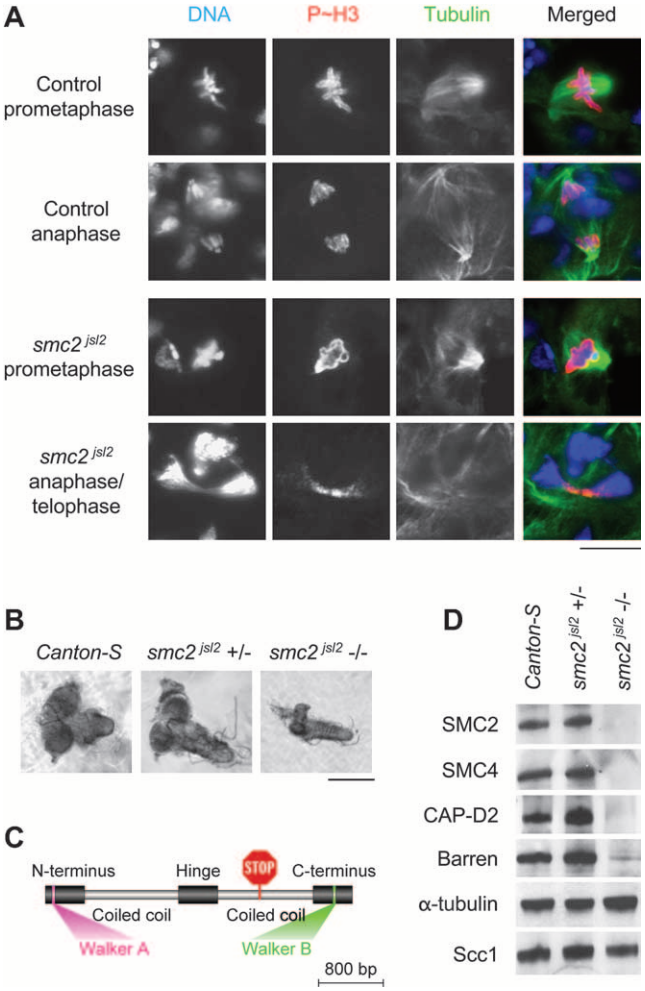

Figure 4.—Phenotypic characterization of the larval lethal *smc2*^{jsl2} mutant. (A) Mitotic figures from heterozygous *smc2*^{jsl2}/*CyO-GFP* controls (above) and homozygous *smc2* ^{jsl2}/*smc2*^{jsl2} second instar larval neuroblasts (below). Brains were squashed and stained for P-H3 (red), α-tubulin (green), and DNA (DAPI, blue). Bar, 10 μm. (B) Brains dissected from wild-type (*Canton-S*), heterozygous *smc2*^{jsl2}/*CyO-GFP* (*smc2*^{jsl2} +/−), and homozygous *smc2* ^{jsl2}/*smc2*^{jsl2} (*smc2* ^{jsl2} −/−) wandering third instar larvae. Bar, 400 μm. (C) Domain structure of SMC2 mapped onto the *smc2* coding sequence, showing the position of the premature stop codon in *smc2*^{jsl2}. (D) Immunoblot analysis of protein extracts from wild-type (*Canton-S*), heterozygous *smc2*^{jsl2}/*CyO-GFP* (*smc2* ^{jsl2} +/−), and homozygous *smc2*^{jsl2}/*smc2*^{jsl2} (*smc2*^{jsl2} −/−) third instar larval brains using antibodies against different condensin subunits, showing how the protein levels of CAP-D2, Barren, and SMC4 are greatly reduced in the absence of SMC2. Approximately two third instar larval brain equivalents were loaded for the wild-type and heterozygous brain extracts and four brain equivalents from the *smc2*^{jsl2} homozygous brain extracts, using α-tubulin as a loading control.

residue 827 caused by a C2482T transition (Figure 4C). This should result in the synthesis of a truncated SMC2 protein lacking the C-terminal globular domain and part of the adjacent coiled-coil sequence, which are expected to be essential for functional activity. However, we were unable to detect a band corresponding to the estimated 94-kDa size in *smc2*^{jsl2} larval or embryonic protein extracts, suggesting that the truncated SMC2 protein may be unstable. As no band corresponding to wild-type SMC2 could be detected in protein extracts from *smc2*^{jsl2} homozygotes, the stability of the remaining condensin components was also tested (Figure 4D). Quantitation of protein levels by phosphorimaging revealed that SMC4 was decreased 6.5-fold, CAP-D2 8.2-fold, and Barren 14-fold compared to wild-type brain extracts, suggesting that the stability of these proteins was greatly affected by the absence of SMC2. By contrast, the Scc1/Rad21 subunit of the cohesin complex was largely unaffected, similar to the α-tubulin loading control.

The effect of condensins on gene expression: Although the various subunits of the condensin complex are clearly essential for proper resolution of chromatids during mitosis (Saka *et al.* 1994; Bhat *et al.* 1996; Lieb *et al.* 1998; Sutani *et al.* 1999; Steffensen *et al.* 2001; Hagstrom *et al.* 2002; Stear and Roth 2002; Hudson *et al.* 2003; Savvidou *et al.* 2005), it seemed plausible that they might also play a role in chromatin behavior during interphase as condensin SMC proteins have long been known to play a role in dosage compensation in *C. elegans* (Chuang *et al.* 1994; Lieb *et al.* 1998). Moreover, chromatin-bridging phenotypes similar to those of *gluon* mutants had been observed in *Su(var)205*^{5} embryos (Kellum and Alberts 1995), which are null mutants for the gene-encoding heterochromatin protein 1 (HP1) with dosage-dependent effects on PEV (Eissenberg *et al.* 1992; Dorn *et al.* 1993). Therefore, we hypothesized that various components of the condensin complex might also be active during interphase, possibly influencing chromatin structure in a manner similar to their effects on mitotic chromosome structure. To explore the possible interphase role of condensin components, the various mutant alleles shown to display a consistent mitotic phenotype were crossed against *white mottled 4* (*w*^{m4h}) flies to examine their effect on PEV, together with reporters of PEV at other loci (Figure 5). The *In(1)w*^{m4h} inversion places the *white* gene (conferring red eye color) near a region of pericentric heterochromatin on the X chromosome (Tartof *et al.* 1984) and suppression of PEV by expansion of euchromatin typically results in increased expression of the *white* gene and thus in a higher proportion of red ommatidia. On the other hand, enhancement of PEV by expansion of heterochromatin leads to decreased expression and more white-eyed flies (Sass and Henikoff 1998). In addition to performing crosses with condensin mutants, established suppressors and enhancers of variegation were also examined to contextually evaluate the strength of effects on PEV seen with the different condensin mutants (aside from the original *glu*^{1} allele, as an intact *white* reporter gene in the *P* element precluded its use in these analyses).

As shown in Figure 6, crosses between *gluon* mutants and *w*^{m4h} females predominantly yielded white-eyed

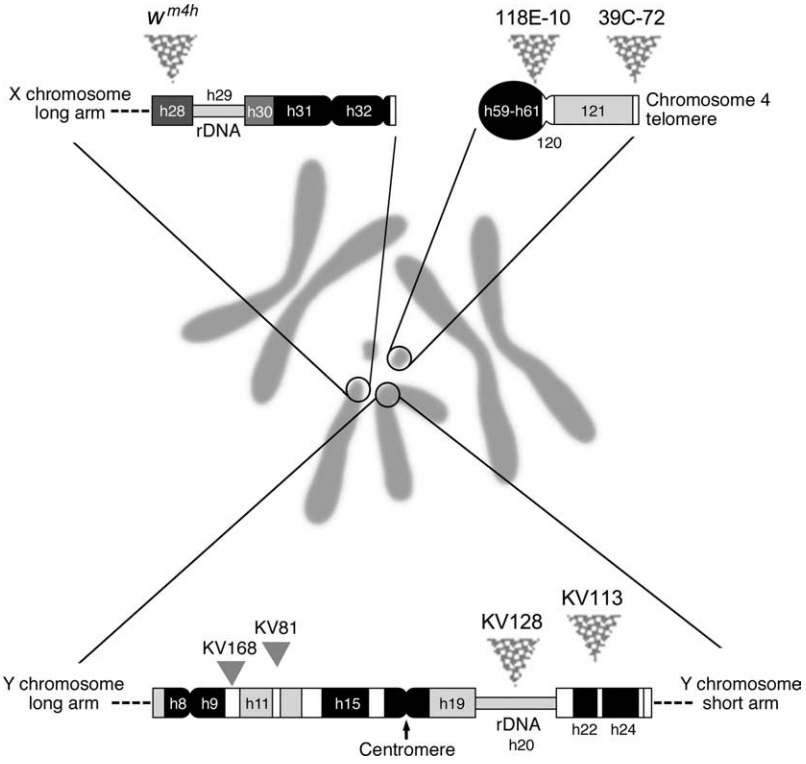

FIGURE 5.—Diagrammatic karyotype of a male *D. melanogaster*, showing the approximate location of the *white* gene in flies carrying the $In(1)w^{m4h}$ inversion on the X chromosome, together with approximate locations of $hsp70\text{-}w^{+}$ transgenes on the fourth chromosome and *SUPor-P* transposon insertions on the Y chromosome. Any heterochromatic *white* reporter genes that appeared sensitive to the dosage of condensin mutant alleles are shown by larger mottled triangles, while neighboring *SUPor-P* transposon insertions on the Y chromosome that appeared unresponsive are shown by the smaller shaded triangles. No obvious effects of condensin mutant alleles on PEV were seen with reporters examined on the second or third chromosomes (not shown).

male progeny for most of the excision alleles, with the exception of $glu^{88\text{-}82}$. Most mutations affecting condensin proteins resulted in strong enhancement of variegation (decreased red eye color) comparable to the dominant enhancer of variegation *Mod(mdg4)* (BÜCHNER *et al.* 2000), suggesting that the wild-type function might be to enhance gene expression by establishing or maintaining an open chromatin conformation. The exceptions included the $glu^{88\text{-}82}$ allele (Figure 6C), which appeared to behave as a strong suppressor of variegation, and the $smc2^{ts12}$ allele (Figure 6B), which had little effect on PEV (possibly because this larval lethal mutation is less severe than those resulting in embryonic lethality). The strong enhancement of variegation due to the $dcap\text{-}g^{64}$ allele differs from the weak suppression of PEV in a w^{m4h} background previously described for $dcap\text{-}g^{K1}$ and $dcap\text{-}g^{K2}$ alleles (DEJ *et al.* 2004). However, the latter alleles contained point mutations that generate premature stop codons in the *dcap-g* coding region, while the D254Y substitution identified in $dcap\text{-}g^{64}$ is predicted to disrupt interactions with other condensin subunits, so differences in the behavior of these alleles may reflect differences in their ability to impair the functional activity of a complex active in interphase. We also observed weak enhancement of variegation with the $barr^{L305}$ allele, in contrast to previous reports that this allele suppresses variegation of w^{m4h} (DEJ *et al.* 2004) and alleviates repression of *mini-white* gene expression due to Polycomb-mediated silencing (LUPO *et al.* 2001). However, our study is the first that we are aware of in which the effects of condensins on interphase gene expression are described using quantitative analyses.

In addition to measuring the effects on PEV by spectrophotometric quantitation of eye pigments (extracted from multiple flies), the frequency of eyes with different levels of pigmentation was also measured by image analysis of individual fly heads. These values were calibrated against the mean eye color suggested by absorbance readings, resulting in the frequency distribution shown in Figure 6D. The frequency distribution reveals that the $glu^{88\text{-}82}$ allele has a bimodal distribution, showing both enhancer and suppressor effects, although overall the mutant behaves as a suppressor of variegation. The behavior of the $glu^{88\text{-}82}$ allele is therefore consistent with its classification as a potential neomorphic mutant (suggested by its sequence), in which opposing effects on gene expression may be caused by both reduced wild-type SMC4 functional activity and translation of a truncated C-terminal protein.

Finally, several alleles of different condensin genes identified as particularly strong enhancers or suppressors of variegation were crossed against each other in a w^{m4h} background to test for possible epistatic effects. Visual inspection of the predominantly red fly eyes in the resulting male progeny initially suggested that suppressors of variegation such as $Su(var)205^{5}$ and $glu^{88\text{-}82}$ might be partially dominant over enhancers of variegation such as glu^{17C}, $dcap\text{-}g^{64}$, and $barr^{L305}$. If $Su(var)205^{5}$ proved to be dominant over glu^{17C}, $dcap\text{-}g^{64}$, or $barr^{L305}$, then this might have suggested that the interphase function of condensins acts upstream of HP1 to

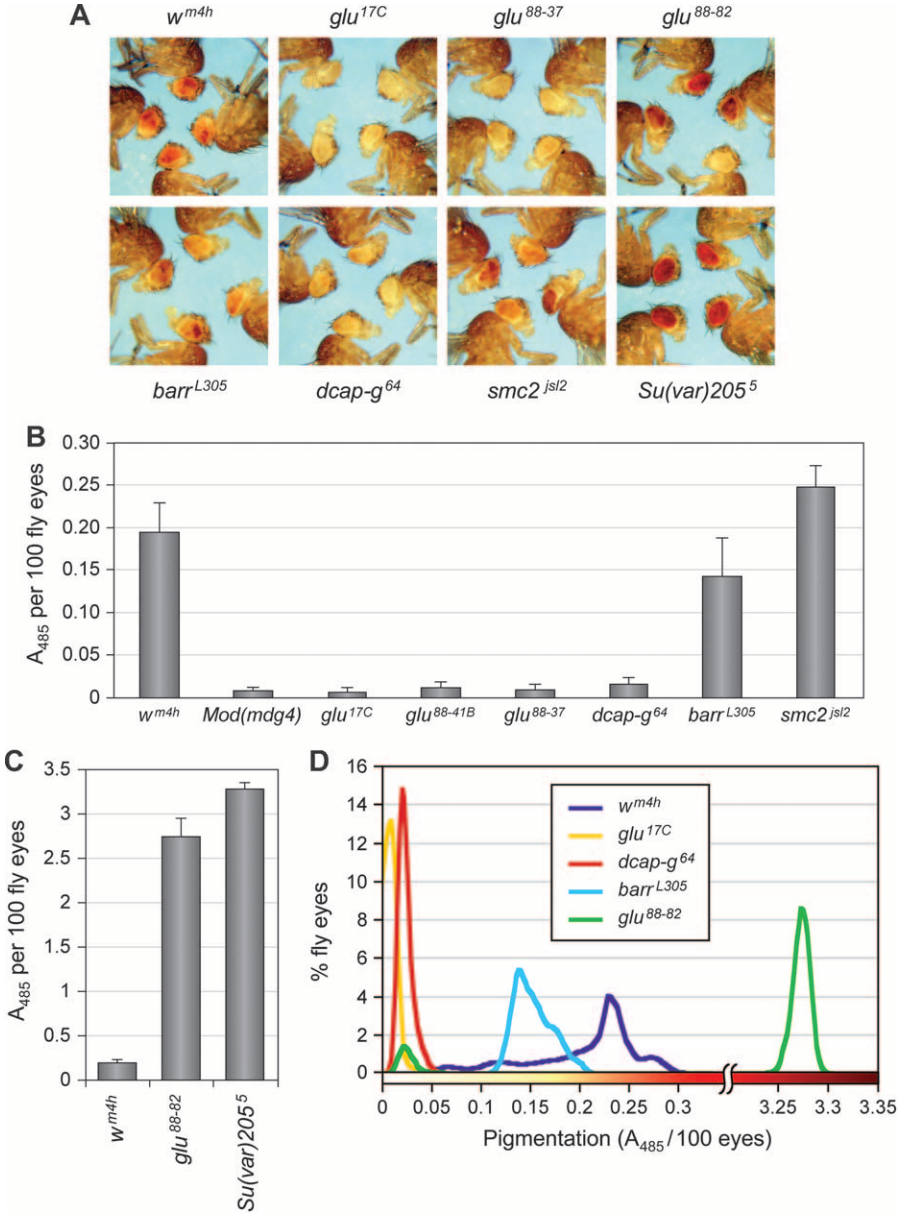

FIGURE 6.—Mutations affecting condensin proteins modify PEV. Virgin females from a w^{mh4} stock were crossed against males from *gluon* excision lines and other condensin mutants. (A) Examples of the resulting male progeny, together with similarly aged w^{mh4} male flies as a control. (B) Absorbance at 485 nm/100 fly eyes in male w^{m4h} flies (control) and male progeny of crosses between w^{m4h} virgin females with male flies carrying mutations affecting various condensin subunits. Values for the *Mod(mdg4)* mutant are also shown for comparison. (C) Absorbance at 485 nm for males heterozygous for $glu^{88\text{-}82}$ compared to $Su(var)205^5$ heterozygotes and w^{m4h} control flies. (D) Distribution of pigment levels in condensin mutant heterozygotes and control flies in a w^{m4h} background. The number of eyes examined for each line were as follows: w^{m4h} control (679 eyes), glu^{17C} (121 eyes), $dcap\text{-}g^{64}$ (451 eyes), $barr^{L305}$ (272 eyes), and $glu^{88\text{-}82}$ (425 eyes).

modulate transcription. Conversely, if these mutant alleles proved to be dominant over $Su(var)205^5$, this might imply that condensin function may be downstream of HP1 activity, possibly reflecting HP1-regulated transcription of condensins as with other cell-cycle regulators (De Lucia *et al.* 2005). However, spectrophotometric measurements revealed intermediate eye pigment levels in these male fly heads (supplemental Figure 1 at http://www.genetics.org/supplemental/), indicating instead that the effect of the different mutant alleles on PEV was approximately dose dependent, with their independent influences combining additively in the assay. Similarly, intermediate eye pigment levels were observed in crosses between either $glu^{88\text{-}82}$ and $dcap\text{-}g^{64}$ or $glu^{88\text{-}82}$ and $barr^{L305}$ in a w^{m4h} background, suggesting that any suppressor effects potentially due to expression of a neomorphic SMC4 C-terminal truncation in $glu^{88\text{-}82}$ were not dominant over the enhancer activity of other condensin mutants, consistent with the observation of both predominantly red- and white-eyed $glu^{88\text{-}82}$ heterozygotes (Figure 6, A and D).

The effect of condensin mutations on PEV at other loci: The effect of the different condensin mutations on PEV was also examined at several other loci, including the bw^D (brown-dominant) allele (Slatis 1955; Henikoff *et al.* 1995), the Sb^V (stubble-variegated) allele (Hayashi *et al.* 1990), and the *Fab-7* repressed *mini-white* reporter gene in *5F24(25,2)* flies (Zink and Paro 1995). However, none of the crosses showed any effect of condensin mutations on PEV. This contrasts with previously published results in which the $barr^{L305}$ allele was shown to suppress *5F24(25,2)* variegation in adult female progeny (Lupo *et al.* 2001) but is nonetheless

consistent with more recent findings concerning the lack of any discernible effect of *dcap-g* mutations on regulation of gene expression by the *Fab-7* Polycomb-group response element (Dej *et al.* 2004). However, as the w^{m4h} inversion places the *white* gene near the *bobbed* multicopy rDNA array within pericentric heterochromatin, it is possible that the PEV assay performed with w^{m4h} flies may be more sensitive to mutations affecting condensin components if such proteins are enriched at rDNA loci as in other species (Freeman *et al.* 2000; Cabello *et al.* 2001; Bhalla *et al.* 2002; Uzbekov *et al.* 2003; D'Amours *et al.* 2004; Wang *et al.* 2004) and since various condensin proteins in Drosophila have been shown to be enriched at mitotic centromeres (Steffensen *et al.* 2001; Savvidou *et al.* 2005). To evaluate whether the effects of condensin mutations on PEV were specific to particular chromosomal regions, additional crosses were performed between selected alleles and flies carrying transgenic *white* reporter genes at different chromosomal loci.

The potential specificity of effects at rDNA-proximal loci was further explored by performing crosses between selected *gluon* alleles and flies carrying insertions of the *SUPor-P* transposon at various intervals on the Y chromosome (Yan *et al.* 2002), which carries a *white* reporter gene flanked by *su(Hw)*-binding regions (Roseman *et al.* 1995). Interestingly, no effect on PEV was seen with KV168 (J448) or KV81 (C882) lines, in which the *SUPor-P* transposon, respectively, maps to the h10 and h11–13 Y chromosomal "h" bands, although each of the different gluon alleles tested was shown to affect PEV in the KV128 (D285) and KV113 (B947) lines, in which the *SUPor-P* transposon, respectively, maps to the h20 and h22–24 Y chromosomal "h" bands and thus relatively close to the *Y-bobbed* rDNA locus (h20–21). In the case of crosses performed with the KV128 insertion (which maps closest to the rDNA locus), the glu^{17C} and $glu^{88\text{-}37}$ alleles clearly suppressed variegation of the *white* reporter gene in the *SUPor-P* transposon, although suppression of variegation was evidently weaker with the $glu^{88\text{-}82}$ allele (Figure 7, A and B). By contrast, each of the different gluon alleles was found to enhance variegation in crosses with KV113 flies (Figure 7C), with no pigmented ommatidia observed in heterozygous mutant progeny. Together, these results further suggest that the rDNA proximal loci may be more sensitive to the effect of condensin mutations.

To test whether the effects of condensin mutations on PEV were exclusive to rDNA proximal loci on the sex chromosomes, additional crosses were performed between selected alleles and flies carrying autosomal centromere- and telomere-proximal $hsp70\text{-}white^{+}$ transgenes (Wallrath and Elgin 1995; Cryderman *et al.* 1999). However, we failed to detect obvious effects on $hsp70\text{-}white^{+}$ expression with pericentric transgene insertions on the second or third chromosome (39C-4 and 118E-12), although derepression of an additional *hsp26-pt-T* reporter transgene has been reported in crosses between these lines and $Su(var)205^{2}$ (Wallrath and Elgin 1995). In agreement with previous findings for $Su(var)205^{2}$ (Wallrath and Elgin 1995; Cryderman *et al.* 1999), we also did not detect effects on PEV for telomeric transgene insertions on both arms of the second chromosome (39C-5 and 39C-27), observing only effects on PEV of both $Su(var)205^{5}$ and condensin mutants with insertions on the fourth chromosome. As shown in Figure 8, each of the *gluon* alleles and the $dcap\text{-}g^{64}$ allele enhanced PEV in crosses with 118E-10 flies, in which the $hsp70\text{-}white^{+}$ transgene is inserted near the centromere of the fourth chromosome (Wallrath and Elgin 1995; Sun *et al.* 2000). Enhancement of variegation was often more readily apparent in female progeny (Figure 8A), possibly because the Y chromosome is itself known to be the most potent suppressor of PEV (Maggert and Golic 2002). Consequently, spectrophotometric comparisons of pigment levels were performed using only male fly heads to more sensitively detect differences due to enhancement of variegation (Figure 8B). By contrast, strong suppression of variegation was observed following crosses with $Su(var)205^{5}$ flies, while the eye pigmentation of progeny heterozygous for $barr^{L305}$ did not appear significantly different from that of controls. Similarly, each of the *gluon* alleles and the $dcap\text{-}g^{64}$ allele enhanced PEV in crosses with 39C-72 flies (Figure 9), in which the $hsp70\text{-}white^{+}$ transgene is inserted near the telomere of the fourth chromosome (Cryderman *et al.* 1999). As with hemizygotes for the 118E-10 $hsp70\text{-}white^{+}$ insertion, enhancement of variegation was more readily apparent in the 39C-72 female progeny (Figure 9A). Although the eyes of progeny heterozygous for $barr^{L305}$ did not generally differ significantly in appearance from those of controls, weak enhancement of variegation was nevertheless apparent by spectrophotometric analysis of homogenized male fly heads (Figure 9B).

DISCUSSION

Condensins are essential for mitotic progression at various stages: The glu^{1}, $glu^{88\text{-}82}$, and glu^{17C} homozygotes all have reduced levels of mitosis and a lower ratio of mitosis to apoptosis compared to wild-type individuals, suggesting that many of these mitotic nuclear divisions may be defective. In particular, the $glu^{88\text{-}82}$ homozygous embryos displayed chromatin bridges in the majority of cells reaching anaphase, as well as a pronounced accumulation of cells in prometaphase and metaphase. Although the sequence data suggest that the $glu^{88\text{-}82}$ allele might be a neomorphic mutant (expressing a truncated SMC4 protein lacking the first 194 amino acids), the observed segregation defects are not unique to this allele and the high frequency of segregation defects observed in $glu^{88\text{-}82}$ mutant embryos can be reproduced in glu^{1} mutants by increasing the mitotic activity in postblastoderm embryos. Finally, the same

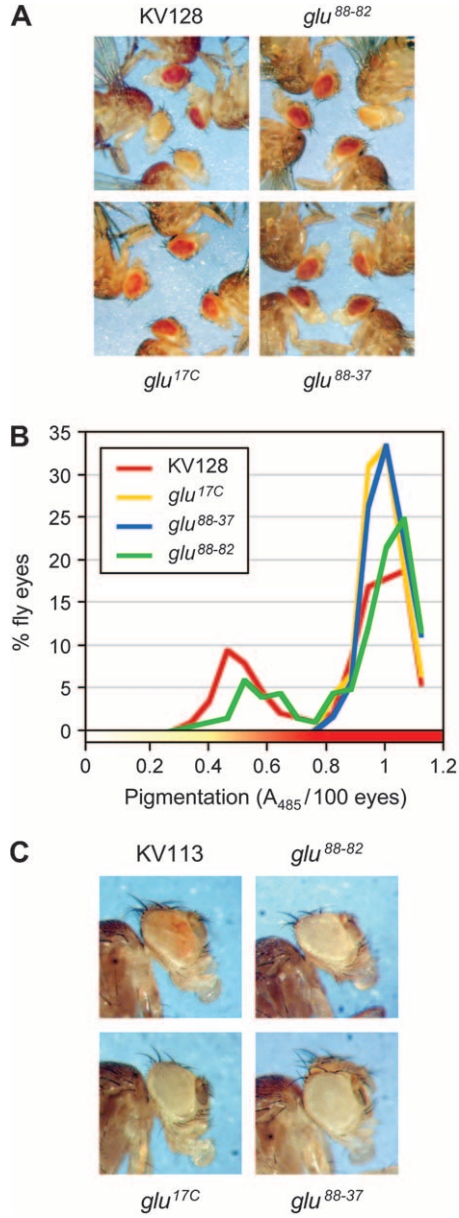

FIGURE 7.—Suppression and enhancement of PEV on the Y chromosome. (A) Examples of the male progeny resulting from crosses between KV128 males and *gluon* virgin females. (B) Distribution of pigment levels in male progeny, comparing the spectrum of eye color in *gluon* heterozygotes with controls hemizygous for the KV128 insertion. The number of eyes examined for each line were as follows: KV128 control (203 eyes), glu^{17C} (124 eyes), $glu^{88\text{-}37}$ (126 eyes), and $glu^{88\text{-}82}$ (206 eyes). (C) Examples of the male progeny resulting from crosses between KV113 males and *gluon* virgin females. The mean and median number of pigmented ommatidia were found to be 10.2 ($\pm$ σ = 17.5) and 3, respectively, in control progeny but the number of pigmented ommatidia was consistently zero for each of the *gluon* mutant heterozygotes.

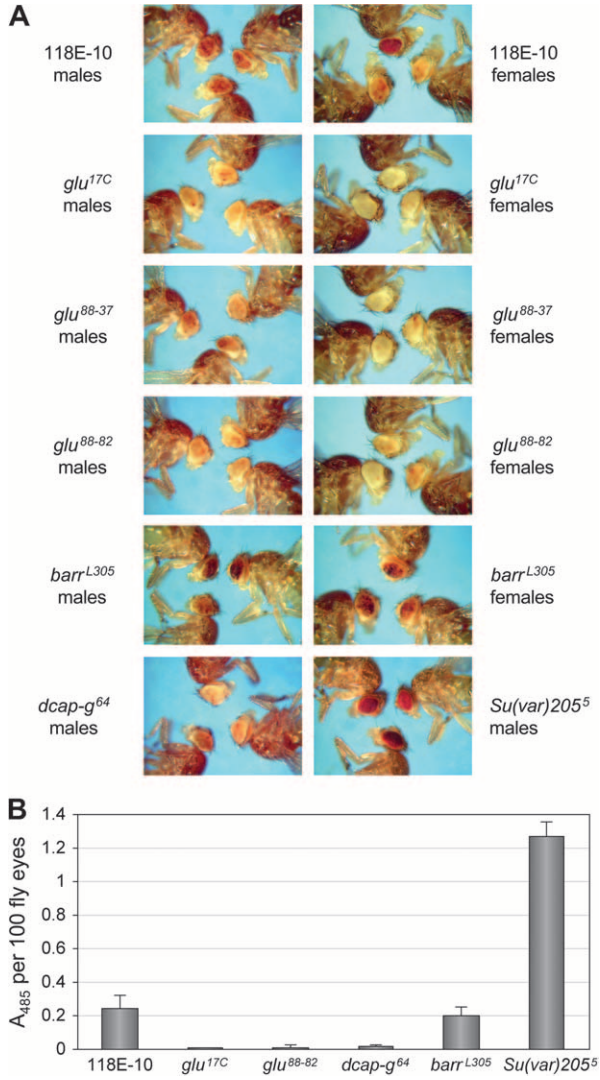

FIGURE 8.—Mutations affecting condensin proteins modify PEV at pericentric heterochromatin on the fourth chromosome. (A) Progeny resulting from crosses between 118E-10 and various condensin mutants or controls. (B) Absorbance at 485 nm/100 fly eyes in male progeny of crosses with 118E-10 females.

chromosome segregation defects have also been observed in glu^{2} and glu^{1}/glu^{2} larval neuroblasts (STEFFENSEN *et al.* 2001) as well as in the $barr^{L305}$ allele (BHAT *et al.* 1996), various *dcap-g* alleles (DEJ *et al.* 2004; JÄGER *et al.* 2005), and depletion of condensin subunits by RNA interference (COELHO *et al.* 2003; SAVVIDOU *et al.* 2005). Therefore, it is clear that condensin is critically required for the orderly segregation of sister chromatids during mitosis in Drosophila. Although it was previously reported that pericentric heterochromatin appeared longer in neuroblasts of *dcap-g* mutant larvae (DEJ *et al.* 2004), measurements of the chromosome arm lengths in glu^{2} larval neuroblasts showed that glu^{2} mutant chromosomes were the same length as those from wild-type larvae (STEFFENSEN *et al.* 2001) and we did not observe any clear difference in mitotic chromosome lengths for any of the condensin mutant alleles described here. In addition, we have not observed any differences in the overall structure or banding pattern of polytene chromosomes while attempting to uncover the potential impact on interphase function of larval lethal condensin

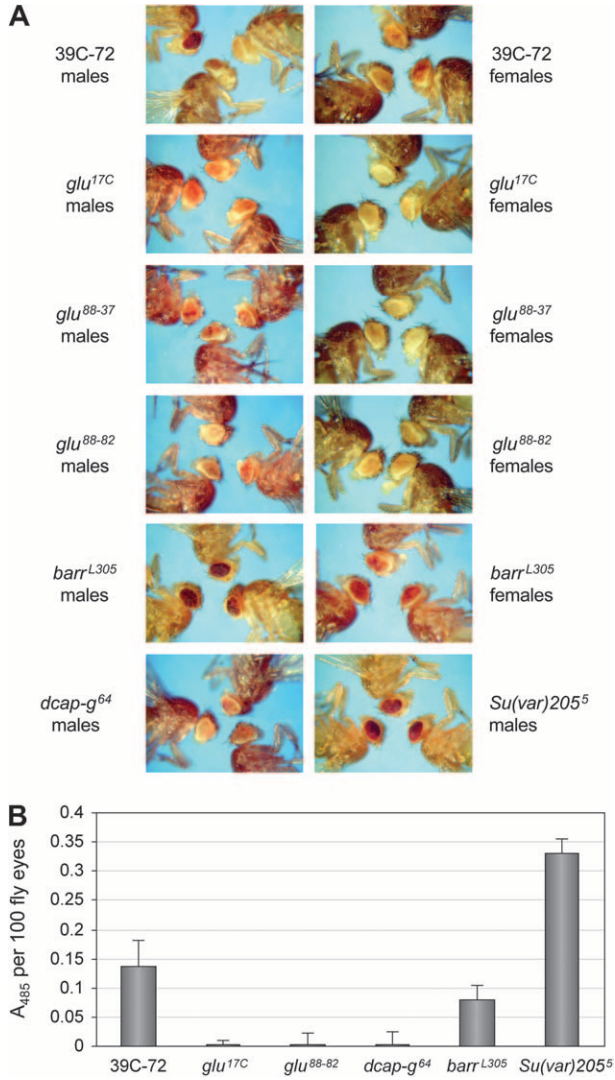

FIGURE 9.—Mutations affecting condensin proteins modify telomeric PEV on the fourth chromosome. (A) Progeny resulting from crosses between 39C-72 and various condensin mutants or controls. (B) Absorbance at 485 nm/100 fly eyes in male progeny.

mutations, such as *glu*^{2} (STEFFENSEN *et al.* 2001) or *smc2*^{isl2} (supplemental Figures 2 and 3 at http://www.genetics.org/supplemental/). It therefore appears that the failure of mitotic chromosomes to resolve in condensin mutants results from a lack of orderly architectural changes, rather than from a complete absence of chromosome condensation *per se* (STEFFENSEN *et al.* 2001; LAVOIE *et al.* 2002; CUVIER and HIRANO 2003; HUDSON *et al.* 2003).

It is also worth noting that mitotic spindles appear normal in both *gluon* and *dcap-g*^{64} mutant embryos, in contrast to the spindle defects observed with different condensin mutations in *S. cerevisiae* (LAVOIE *et al.* 2000, 2002; OUSPENSKI *et al.* 2000). Normal mitotic spindles are also observed in the Drosophila *barr*^{L305} mutant (BHAT *et al.* 1996), chicken DT40 cells depleted of SMC2 (HUDSON *et al.* 2003), and SMC4 depleted by RNAi in *C. elegans* (HAGSTROM *et al.* 2002), so it appears that the condensin proteins do not affect spindle assembly in metazoans. Therefore, the segregation defects in *gluon* mutants do not appear to be due to defective spindle formation but are more consistent with a role for condensins in preventing chromosome entanglement. By contrast, abnormal mitotic spindles were frequently observed in the *smc2*^{isl2} allele, although this might be partly explained by the difficulty in preparing neuroblast squashes from significantly smaller larval brains.

Consistent with our previous findings (STEFFENSEN *et al.* 2001), quantitation of the mitotic phenotypes in condensin mutant embryos suggests that most cells are delayed in prophase/prometaphase and metaphase, whereas the majority of cells reaching anaphase display chromatin bridges. The observation that embryonic lethal *gluon* and *dcap-g* alleles accumulate in metaphase while larval alleles do not is possibly because the embryonic lethal alleles are more severe (STEFFENSEN *et al.* 2001). On the other hand, the absence of any similar mitotic delay in cultured cells depleted of condensin components (COELHO *et al.* 2003; HUDSON *et al.* 2003) might reflect checkpoint differences in cultured cells from various species, as a metaphase delay together with chromatin bridges has also been observed in *C. elegans mix-1* mutant embryos, in which the ortholog of SMC2 is affected (LIEB *et al.* 1998). A roughly threefold greater number of prometaphase figures was previously reported for *dcap-g* mutant embryos compared to wild type (DEJ *et al.* 2004), although different criteria were used to classify prometaphase figures in this study, with only the morphology of the chromosomes (but not the mitotic spindle or nuclear lamina) scored for the purposes of quantitation. Nevertheless, our analyses based on consistent classification of both mitotic spindle and chromosome behavior confirm the accumulation of mitotic cells in prophase/prometaphase in both embryonic lethal *gluon* and *dcap-g* alleles. On the basis of our observations and previous findings that mitotic chromosome condensation is delayed but not abolished in condensin mutants (STEFFENSEN *et al.* 2001; HUDSON *et al.* 2003), we speculate that condensation checkpoints may exist in intact cells, similar to previously reported topoisomerase II and DNA damage checkpoints (DOWNES *et al.* 1994; RIEDER and Cole 1998; GIMÉNEZ-ABIÁN *et al.* 2002; MIKHAILOV *et al.* 2004) or possible monitoring of kinetochore tension through the spindle checkpoint (MUSACCHIO and HARDWICK 2002). If so, the presence of such a checkpoint may prevent the most extreme condensation defects from occurring, so segregation defects are the main phenotypes usually observed in whole organisms with reduced condensin activity (LIEB *et al.* 1998; STEFFENSEN *et al.* 2001; HAGSTROM *et al.* 2002; STEAR and ROTH 2002). By contrast, more extreme chromosome structural defects or compromised kinetochore–microtubule interactions may be apparent only in cultured cells from

TABLE 1

Summary of effects of *gluon* alleles on PEV with different reporters

Reporter	Chromosomal location	SMC4 mutant allele		
		$glu^{88\text{-}82}$	$glu^{88\text{-}37}$	glu^{17C}
w^{m4h}	X (rDNA proximal)	+++++	−	−
KV128	Y (nearest rDNA)	+	++	++
KV113	Y (rDNA proximal)	−	−	−
118E-10	Fourth (pericentric)	−	−	−
39C-72	Fourth (telomeric)	−	−	−

Relative expression of the *white* reporter is indicated by a plus sign for increased eye pigmentation relative to controls (suppression of variegation) or a minus sign for decreased eye pigmentation (enhancement of variegation). Among the other condensin components, the $dcap\text{-}g^{64}$ allele appeared to show effects on PEV similar to those seen with $glu^{88\text{-}37}$ and most other *gluon* mutant alleles tested, whereas relatively modest effects on PEV were observed with the $barr^{L305}$ allele and little or no differences were observed between $smc2^{jsl2}$ heterozygotes and controls.

different species (Coelho *et al.* 2003; Hudson *et al.* 2003; Hirota *et al.* 2004; Ono *et al.* 2004; Savvidou *et al.* 2005).

Unfolding the role of condensins during interphase: In contrast to previous reports consistently describing condensins primarily as suppressors of variegation (Lupo *et al.* 2001; Dej *et al.* 2004), we have found that the same mutant alleles can promote either suppression or enhancement of variegation at different loci on the same or different chromosomes (Table 1). The observation that most mutations affecting condensin proteins resulted in strong enhancement of variegation at the w^{m4h} locus was initially surprising, as one might expect that proteins that compact DNA would encourage expansion of heterochromatin, resulting in suppression of variegation as previously reported (Bhat *et al.* 1996; Dej *et al.* 2004). Nevertheless, one should also bear in mind the possibility that different condensin proteins may not function in the same way or as members of the same complex during interphase, consistent with the contrasting requirements for different condensin proteins during interphase in *S. pombe* (Aono *et al.* 2002) or during silencing at mating-type loci in *S. cerevisiae* (Bhalla *et al.* 2002). Indeed, different proteins are already known to be associated with the SMC2/4 heterodimer in *C. elegans* that participates in dosage compensation (Chuang *et al.* 1994; Lieb *et al.* 1996, 1998). We recently verified the existence of a canonical condensin I complex in Drosophila embryos (Savvidou *et al.* 2005) but it is still unknown what components an interphase condensin complex might contain in flies. Although the various condensin proteins are enriched in actively dividing tissues such as larval brains (Steffensen *et al.* 2001; Savvidou *et al.* 2005), we were unable to detect these proteins in equivalent amounts of extracts prepared from nondividing tissues such as third instar larval salivary glands (supplemental Figure 4 at http://www.genetics.org/supplemental/). However, it is possible that the interphase activity of condensin proteins may be specific to particular tissues or developmental stages and may require comparatively low protein levels to exert significant epigenetic effects.

Although we do not yet know the identity of sequence elements that might mediate the effects of condensins on gene expression, it is striking that we observed effects only with reporters of variegation close to the rDNA locus on the X and Y chromosomes and also on the fourth chromosome (Figure 5), as condensin proteins were shown to be enriched at rDNA loci in *S. cerevisiae* (Freeman *et al.* 2000; Bhalla *et al.* 2002; D'Amours *et al.* 2004; Wang *et al.* 2004) and localized to nucleoli during interphase in Xenopus cells (Cabello *et al.* 2001; Uzbekov *et al.* 2003). Among the various heterochromatic regions to which condensins bind preferentially during mitosis in *S. cerevisiae* (Wang *et al.* 2005), a functional connection with rDNA is also suggested by the cohesin-dependent role of condensins in the establishment of rDNA condensation (Lavoie *et al.* 2002, 2004). Furthermore, the additional recruitment of condensins to the rDNA locus in anaphase (dependent on Ipl1/aurora B kinase and Cdc14 phosphatase activity) is specifically required to mediate rDNA condensation and resolution (Freeman *et al.* 2000; Bhalla *et al.* 2002; D'Amours *et al.* 2004; Lavoie *et al.* 2004; Sullivan *et al.* 2004; Wang *et al.* 2004).

The apparent specificity of condensin-mediated epigenetic phenomena to the fourth chromosome is intriguing. The fourth chromosome of Drosophila is unlike other autosomes but similar to the X or Y chromosomes insofar as flies haploid for these chromosomes are viable, albeit sterile in the case of XO males and usually sterile in the case of haplo-4 flies (Lindsley and Grell 1968). The fourth chromosome is also unusual among the autosomes as it is predominantly heterochromatic, although it also contains interspersed euchromatic and heterochromatic domains (Sun *et al.* 2000, 2004), whereas domains of constitutive heterochromatin are normally limited to pericentric and telomeric DNA in most metazoans. Similarly, the Y chromosome contains over three times more pericentric heterochromatin than either the second or the third chromosome in Drosophila (Peacock *et al.* 1978; Adams *et al.* 2000). The behavior of telomeric gene silencing on the fourth chromosome differs from that of other autosomes and appears to more closely resemble the general pattern of pericentric gene silencing dependent on HP1 (Cryderman *et al.* 1999; Sun *et al.* 2004). Other similarities between the fourth chromosome and the X chromosome seem to be revealed by the chromosomal specificity of painting of fourth (POF) binding in different Drosophila species. For example, the POF protein binds only the fourth chromosome in

D. melanogaster males but decorates the entire X chromosome exclusively in *D. busckii* males (Larsson *et al.* 2004). Moreover, POF binding to the male X chromosome (as well as to the fourth chromosome) in species such as *D. ananassae* and *D. malerkotliana* has been shown to colocalize with MSL3, suggesting a relationship between sex-linked dosage compensation and a protein associated with the fourth chromosome (Larsson *et al.* 2004). Finally, it appears that the fourth chromosome may pair with the X in meiosis as persistent associations between pericentric heterochromatin of the X chromosome and the fourth chromosome have been described in prophase I oocyte nuclei (Dernburg *et al.* 1996), while triplo-4 causes an increased frequency of X chromosome nondisjunction (Sandler and Novitski 1956). Similarly, duplications of the X chromosome can interfere with segregation of either the fourth chromosome or the nonexchange X chromosomes (Hawley *et al.* 1992). On the basis of these various similarities, we envisage that the apparent chromosomal specificity of interphase condensin activities might reflect recognition of a particular chromatin structure or sequence elements common to heterochromatin of the fourth chromosome and rDNA-proximal loci on the sex chromosomes. Although such specific chromosomal targets remain to be identified, it is possible that these might include the *Hoppel* element/*1360* transposon, as this appears to be associated with heterochromatin formation on the fourth chromosome (Cryderman *et al.* 1999; Sun *et al.* 2004) and also appears to be enriched in X chromosomal pericentric heterochromatin (Bartolomé *et al.* 2002).

Strikingly, we have observed both strong suppression and enhancement of variegation at the *white* gene in a w^{m4h} background in the case of the $glu^{88\text{-}82}$ allele. By contrast, the other *gluon* mutant alleles and $dcap\text{-}g^{64}$ behaved only as strong enhancers of variegation at this locus, even though each of these other mutations yielded similar mitotic phenotypes to $glu^{88\text{-}82}$. If the $glu^{88\text{-}82}$ allele proves to be a neomorph as suggested by sequence data, then the suppression of variegation caused by this allele might be explained in terms of the activity of a C-terminal truncated protein. Nevertheless, the effect of $glu^{88\text{-}82}$ on PEV at other loci shows enhancer or suppressor behavior similar to that of other *gluon* alleles. We therefore postulate that condensins might affect gene silencing by regulating the spread of heterochromatin, possibly by exerting effects on chromatin boundary elements or insulators (Lupo *et al.* 2001; Cuvier *et al.* 2002). In the absence of full condensin function, gene expression might be either enhanced or repressed as developmentally regulated patterns of gene expression become locked (Pirrotta 1997; Francis and Kingston 2001; Ringrose and Paro 2004). It is also possible that the contrasting effects of the $glu^{88\text{-}82}$ allele on PEV at a given locus might reflect the relative ability of a truncated SMC4 protein to interact with distinct subunits of the condensin I or condensin II complex, as non-SMC subunits of these different complexes in vertebrates display distinct localization patterns and appear to contribute differently to mitotic chromosome architecture (Ono *et al.* 2003, 2004). Similarly, differential behavior of distinct SMC4-containing condensin complexes at different loci might explain the contrasting effects of both $glu^{88\text{-}82}$ and other *gluon* alleles at different loci on the Y chromosome. However, relatively little is currently known about the behavior of the condensin II complex in Drosophila, aside from a possible role for the CAP-D3 subunit in male meiosis (Savvidou *et al.* 2005). Consequently, further analysis of the interplay between different condensin proteins and the transcriptional machinery will be required to elucidate mechanistic details of their potential involvement in epigenetic phenomena. Intriguingly, condensin mutants in *S. cerevisiae* were shown to relocalize telomeric Sir2p to the centromere proximal rDNA and displayed histone hyperacetylation at telomeres (Machín *et al.* 2004), while deletion of *SIR2* increased instability of rDNA by impairing association of the Scc1 cohesin subunit (Kobayashi *et al.* 2004). As the *sir2* gene appears to be similarly nonessential for viability in Drosophila yet has minor effects on position-effect variegation (Åström *et al.* 2003), it is tempting to speculate that the effects of condensins on gene expression might be also mediated at least in part by SIR2 in this species.

In conclusion, it remains to be seen exactly how condensins might behave differently to facilitate interconversion between different states of chromatin compaction in interphase and to compact chromatin in mitosis. The current observations, however, can be unified if one postulates that condensin organizes a particular type of chromatin, which is then acted upon by other components to either bring about chromosome condensation in mitosis or regulate gene expression at particular loci. It is therefore clear that there is still much to discover about how these proteins might operate at various stages of the cell cycle and in different developmental contexts.

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